Estrous cycle and cold stress in iron-deficient rats

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Whether iron-deficient female rats can thermoregulate when exposed to cold was studied to assess the interactive effects of iron deficiency and the female reproductive cycle. Rats were assigned to either iron-deficient (~3 ppm Fe, n = 60) or control (35 ppm Fe, n = 60) diets for approximately 5 weeks. The two groups were then subdivided into five groups, four based on stage of the estrous cycle (proestrus, estrus, metestrus, diestrus) and the fifth group was ovariectomized. Animals were exposed to 4° C for 6 hours. Following sacrifice, tissues were collected for analysis of thyroid hormone and iron status indices. There was an interactive effect of iron status and the estrous cycle on core temperature response to the cold ($P \leq 0.05$). Iron-deficient animals had lower core temperatures following cold exposure than did controls, and this effect was most pronounced during proestrus. Ovariectomy resulted in significantly lower core temperatures following cold stress as well as lower plasma thyroid hormone concentrations. Plasma thyroxine concentrations were unaffected by iron status or the estrous cycle, and plasma triiodothyronine concentrations were lower ($P < 0.001$) in the iron-deficient rats than in controls. Thyroxine monodeiodinase activity in the liver was lower ($P \leq 0.01$) in iron-deficient animals than in controls; this conforms with the plasma triiodothyronine findings. Brown adipose tissue deiodinase was not affected by either iron status or the estrous cycle. In conclusion, iron deficiency impairs thermoregulation in female rats, and this effect is related to the ovarian cycle. It was not, however, attributable to estrous cycle effects on indices of thyroid hormone metabolism.

Keywords: thyroid hormones; thyroxine monodeiodinase; thermogenesis; temperature regulation

Introduction

The thyroid hormone system plays a key role in thermoregulation in mammals.[1] The production of active hormone, triiodothyronine (T_3), from precursor, thyroxine (T_4), is regulated by the enzyme thyroxine monodeiodinase.[2] There are two principal forms of thyroxine monodeiodinase: type I deiodinase, which is found primarily in the liver and kidney; and type II deiodinase, which is located in the central nervous system and brown adipose tissue.[2] Although altered thyroid function results in impaired thermoregulatory performance, many factors can affect thyroid function. These include genetic,[3] environmental,[4,5] physiological,[6-9] and dietary/nutritional factors.[10-17] The type II deiodinase and brown adipose tissue in general have both been demonstrated as key components of thermoregulation in rodents.[18,19] The brown adipose deiodinase is also regulated by many of the same factors which influence thermogenesis; eg, environmental,[3,20,21] genetic,[3] hormonal[21] and nutritional factors.[16,17]

Iron deficiency lowers plasma thyroid hormone concentrations[16,17,22-25] and tissue deiodinase activity[16,17] in male rats maintained in a 24° C environment. The thyroid indices and thermoregulatory response to cold exposure[23-25] are also lower in iron-deficient rats than in controls. Similar studies in iron-deficient female rats at room temperature reveal no differences in plasma total T_3 concentration despite depressed hepatic type I deiodinase activity.* Further studies of iron-deficient female rats at 24° C suggest that the iron-deficient thyroid response to the ovarian steroids

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is altered when compared to controls.† Estradiol treatment increased hepatic type I deiodinase activity in female iron-sufficient controls and decreased activity in female, iron-deficient animals;† this, in part, apparently explains the altered estrual cyclicity of deiodinase activity in iron-deficient female rats.*

The female reproductive cycle is known to affect the thyroid hormone system.[7,26,27] Thyroid and thermogenic indices are influenced by the estrous cycle,[28] ovariectomy,[29] and ovarian steroid replacement.[8,9] Based on this information and the sex-related differences in thyroid/thermoregulatory function in iron deficiency, we conducted the following study to assess the thermoregulatory response of iron-deficient female rats during acute cold exposure, and to determine if thermoregulatory function was dependent on the stage of the estrous cycle. We have also examined iron-deficient and iron-sufficient animals following ovariectomy to determine any direct effects of the ovarian cycle and to assess its relative importance in temperature regulation during acute cold exposure.

Materials and methods

Figure 1 shows the experimental design of the current study. Weanling, female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) were randomly assigned to either iron-deficient (ID, ~3 ppm iron, n = 60) or iron-sufficient (CN, 35 ppm iron as ferrous sulfate, n = 60) diets. The diet formulation was based on the recommendations of the American Institute of Nutrition;[30] ie, 70% carbohydrate, 20% casein, 5% corn oil, 35% mineral mix, 1% vitamin mix, 0.5% choline and methionine. The dietary carbohydrate source was corn starch;[31] cellulose was omitted because of its variable iron content. Animals were given distilled deionized water ad libitum and were housed individually in stainless steel cages in a climate controlled room with a 12 hr light:dark cycle (lights on at 0600 hr).

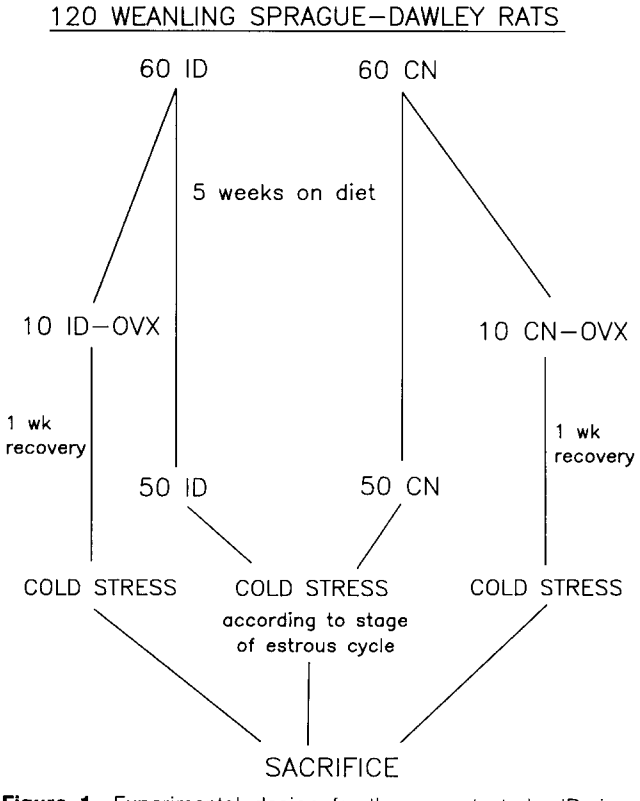


Figure 1 Experimental design for the current study. ID, iron-deficient; CN, control; OVX, ovariectomized; wk, week.

Following 3 weeks of dietary treatment, the stage of the estrous cycle was determined by the vaginal smear technique. Smears were obtained daily by vaginal lavage at 0800 hr. Slides were air-dried, stained (Leukostat Stain Kit, Fisher Scientific, Orangeburg, NY, USA), and observed under a light microscope for discrimination of cell types and subsequent assignment of cycle stage (proestrus, P; estrus, E; metestrus, M; diestrus, D). Inter-reader reliability of assignment of stage was >90% and only rats having two or more complete cycles were included.

Two subgroups of rats (10 ID, 10 CN) were bilaterally ovariectomized (OVX) under ether anesthesia. One ID animal died during surgery from over-anesthetization, another ID died following complications unrelated to treatment. Animals were allowed to recover for 7 days prior to sacrifice, at which time CN-OVX animals were 115 ± 3% (mean ± SD) of surgical weight, compared to 106 ± 4% for ID-OVX rats.

During weeks 4 and 5 of dietary treatment, animals that had demonstrated at least two complete estrous cycles and OVX groups were acutely cold stressed for a period of 6 hours (beginning at 1000 hr). Animals did not have access to food during the cold stress. There were 8–14 animals per treatment group, as noted throughout the tables and results section. Baseline rectal temperatures were obtained by using a rectal thermistor (YSI Series 700 Thermo-Sistor, YSI Co., Yellow Springs, OH, USA), then rats were placed in an environmental chamber at 4° C. Food was not provided during the cold stress. Rectal temperatures were obtained each hour. Fifteen to eighteen animals were cold stressed daily with treatment groups randomized throughout the experiment; both ID and CN animals averaged 32 days of treatment at the time of cold stress/sacrifice, the ovariectomized animals were slightly older (average, 36 d for ID and 37 d for CN). To have the average length of cold exposure 6 hours, animals were killed between 5½ and 6½ hr. Thus, statistical analysis of cold exposure data was through the 5 hr time point to include all animals; however, metabolic responses (eg, plasma thyroid hormones) indeed reflect a 6 hr cold exposure.

Animals were killed by decapitation. Trunk blood was collected into heparinized tubes from which plasma was obtained following centrifugation. Tissues were removed rapidly and processed as described below. Hemoglobin and hematocrit were determined by a Coulter Counter (model #S-Plus IV, Coulter Electronics, Hialeah, FL, USA). Livers were dissected and weighed. The median lobe was removed and processed for mineral content; the remaining lobes were used for preparation of microsomes for determination of deiodinase activity (described below). Tissue mineral content was determined on lyophilized liver sections and spleens. Tissues were digested in nitric acid[32] and analyzed using inductively coupled plasma emission spectroscopy.[33]

Thyroxine monodeiodinase activity was determined on microsomal fractions of liver based on a modification of a previously published method[34] and based on the release of $^{125}I^-$ from [^{125}I]-thyroxine (Amersham Corp., Arlington Heights, IL, USA). Isotopically labeled T_4 was purified daily using Sephadex LH-20 column chromatography to less than 1% contaminating iodide. Microsomal samples were incubated in phosphate buffer containing dithiothreitol (3 mmol/L) and thyroxine (~2–11 μM) for 20 minutes. The reaction was terminated by the addition of human serum and trichloroacetic acid. Released iodide was separated from substrate by using Dowex ion exchange resin (Dowex 50W–X2, 100–200 mesh).[34] Samples were counted in a

Packard gamma counter to a 1% σ error, and data expressed in double reciprocal plots for subsequent estimation of V_{max} and apparent K_m.

Interscapular brown adipose tissue (IBAT) was homogenized in 2 mL 0.25 M sucrose, 1 mmol/L HEPES buffer. Infranatants were separated after centrifugation for 15 minutes at 200*g* at 4° C. Type II deiodinase activity was estimated in IBAT infranatant as in the liver, with the exception that 1 mmol/L propylthio-uracil was added to the incubation to inhibit any type I deiodinase present. Samples were run in quadruplicate at one substrate concentration (~13 nmol/L T_4). All deiodinase data are expressed per mg protein as determined by a modified Lowry procedure (Sigma Kit #P5656).

Plasma thyroid hormone concentrations were determined by commercially available kits (Diagnostic Products Corp., Los Angeles, CA, USA). All determinations were made in one assay. The inter- and intra-assay coefficients of variation in our laboratory are <6% and <4%, respectively, for both T_3 and T_4.

Most data were analyzed by using a two-way analysis of variance. The two class variables were iron status (ID, CN) and stage of the estrous cycle (P, E, M, D, OVX). Type III sums of squares were used because of unequal cell sizes. A post-hoc Scheffé test was used to assign differences between groups when the analysis of variance revealed a significant interaction. Core temperature was analyzed by using a three-way analysis of variance with repeated measures (time). A stepwise multiple regression was used to predict core temperature change in order to assess relative importance of individual variables. All statistics were calculated by using the SAS/STAT software (Release 6, SAS Inc., Cary, NC, USA). Significance was assigned to $P \leq 0.05$.

All data, unless otherwise indicated, represent mean ± standard error of the mean. All reagents, unless otherwise noted, were obtained from Sigma Chemical Company (St. Louis, MO, USA). Vitamin-free casein was purchased from ICN Biochemicals (Costa Mesa, CA, USA).

Table 1 Body weight and hematological variables in ID and CN animals after a 6-hour cold stress

	(N)	Body weight (g)	Hemoglobin (g/L)	Hematocrit % PCV
Iron-deficient				
P	(11)	132.9 ± 4.8	50 ± 1	13.7 ± 0.2
E	(12)	142.0 ± 4.5	52 ± 1	14.2 ± 0.5
M	(13)	132.0 ± 4.8	51 ± 2	14.2 ± 0.7
D	(14)	137.6 ± 3.1	52 ± 2	14.7 ± 0.4
OVX	(8)	132.4 ± 6.1	48 ± 2	13.3 ± 0.5
Overall	(58)	135.6 ± 2.0	51 ± 1	14 ± 0.3
Control				
P	(11)	164.4 ± 4.7	134 ± 2	36.2 ± 0.6
E	(14)	158.9 ± 3.2	136 ± 2	36.5 ± 0.6
M	(10)	159.9 ± 3.4	139 ± 2	37.4 ± 0.4
D	(15)	164.1 ± 4.1	132 ± 3	36.5 ± 0.7
OVX	(10)	173.5 ± 2.6	131 ± 1	36.3 ± 0.3
Overall	(60)	163.9 ± 1.7	134 ± 1	37 ± 0.3
Analysis of variance table				
Iron status		0.001	0.001	0.001
Estrous cycle		NS	NS	NS
Iron × cycle		NS	NS	NS

PCV, % packed cell volume; ID, iron-deficient; CN, control; P, proestrus; E, estrus; M, metestrus; D, diestrus; OVX, ovariectomized.
All data are presented as mean ± SEM.

Table 2 Absolute and relative tissue weights in ID and CN animals after a 6-hour cold stress

	(N)	Liver weight g	Liver weight g/kg	IBAT weight g	IBAT weight g/kg
Iron-deficient					
P	(11)	4.98 ± 0.19	37.8 ± 1.6	0.225 ± 0.011	1.70 ± 0.07
E	(12)	5.13 ± 0.21	36.3 ± 1.1	0.217 ± 0.016	1.52 ± 0.08
M	(13)	5.03 ± 0.16	38.3 ± 1.0	0.220 ± 0.011	1.67 ± 0.07
D	(14)	4.99 ± 0.10	36.3 ± 0.4	0.236 ± 0.014	1.73 ± 0.11
OVX	(8)	4.99 ± 0.23	37.7 ± 1.1	0.213 ± 0.011	1.63 ± 0.13
Overall	(58)	5.03 ± 0.07	37.2 ± 0.5	0.223 ± 0.005	1.65 ± 0.04
Control					
P	(11)	5.08 ± 0.16	30.9 ± 0.6	0.240 ± 0.013	1.47 ± 0.09
E	(14)	5.07 ± 0.15	31.9 ± 0.74	0.232 ± 0.008	1.47 ± 0.05
M	(10)	5.10 ± 0.19	31.8 ± 0.68	0.230 ± 0.013	1.44 ± 0.08
D	(15)	5.34 ± 0.12	32.7 ± 0.8	0.241 ± 0.008	1.48 ± 0.07
OVX	(10)	5.71 ± 0.15	32.9 ± 0.7	0.247 ± 0.009	1.43 ± 0.05
Overall	(60)	5.25 ± 0.07	32.1 ± 0.3	0.238 ± 0.004	1.46 ± 0.03
Analysis of variance table					
Iron status		0.02	0.001	0.04	0.001
Estrous cycle		NS	NS	NS	NS
Iron × cycle		NS	NS	NS	NS

ID, iron-deficient; CN, control; P, proestrus; E, estrus; M, metestrus; D, diestrus; OVX, ovariectomized.
All data are presented as mean ± SEM.

Results

Iron-deficient animals weighed significantly less than controls and were severely anemic after the dietary treatment (*Table 1*). Relative liver and IBAT weights were significantly higher in ID animals than in CN (*Table 2*). Liver and spleen iron concentrations were significantly lower in the ID animals (*Table 3*). Liver copper concentrations were higher in ID animals than

Table 3 Liver and spleen mineral contents in ID and CN animals after a 6-hour cold stress

	(N)	Liver iron (μmol Fe/g dry weight)	Liver copper (μmol Cu/g dry weight)	Spleen iron (μmol Fe/g dry weight)	Copper (μmol Cu/g dry weight)
Iron-deficient					
P	(11)	1.6 ± 0.1	0.36 ± 0.03	3.9 ± 0.3	0.25 ± 0.02
E	(12)	1.5 ± 0.1	0.36 ± 0.03	4.0 ± 0.2	0.25 ± 0.02
M	(13)	1.5 ± 0.1	0.36 ± 0.03	3.7 ± 0.1	0.25 ± 0.02
D	(14)	1.5 ± 0.1	0.38 ± 0.03	3.6 ± 0.1	0.27 ± 0.02
OVX	(8)	1.5 ± 0.1	0.35 ± 0.05	3.9 ± 0.2	0.27 ± 0.02
Overall	(58)	1.5 ± 0.1	0.36 ± 0.02	3.8 ± 0.1	0.27 ± 0.01
Control					
P	(11)	12.7 ± 0.7	0.27 ± 0.02	18.9 ± 1.2	0.25 ± 0.03
E	(14)	11.9 ± 0.8	0.27 ± 0.02	20.1 ± 1.5	0.24 ± 0.02
M	(10)	11.9 ± 0.8	0.27 ± 0.02	18.2 ± 1.5	0.25 ± 0.02
D	(15)	11.9 ± 0.4	0.25 ± 0.01	17.1 ± 1.1	0.25 ± 0.02
OVX	(10)	12.0 ± 0.5	0.25 ± 0.02	16.7 ± 1.3	0.22 ± 0.02
Overall	(60)	12.1 ± 0.3	0.27 ± 0.01	18.3 ± 0.6	0.24 ± 0.02
Analysis of variance table					
Iron status		0.001	0.001	0.001	NS
Estrous cycle		NS	NS	NS	NS
Iron × cycle		NS	NS	NS	NS

ID, iron-deficient; CN, control; P, proestrus; E, estrus; M, metestrus; D, diestrus; OVX, ovariectomized.
All data are presented as mean ± SEM.

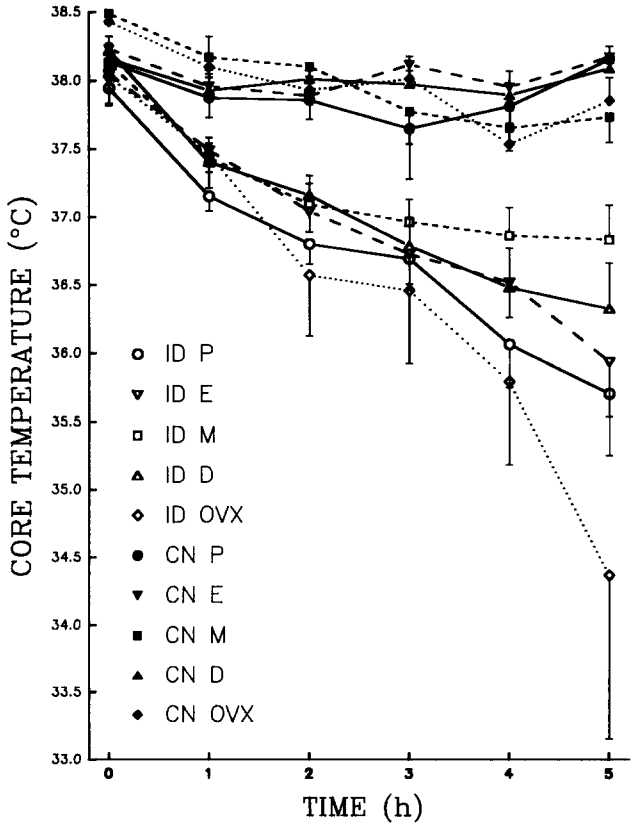


Figure 2 Core temperature response to cold exposure in iron-deficient (ID) and control (CN) rats during each stage of the estrous cycle and in ovariectomized (OVX) ID and CN rats. P, proestrus; E, estrus; M, metestrus; D, diestrus.

in controls; however, spleen copper concentrations were unaffected by iron status.

Figure 2 presents the average core temperatures during the cold exposure for the ten groups. *Figure 3* represents the change in core temperature during the cold stress period ($Temp_{5h} - Temp_{0h}$). There was a significant three-way interaction among time, iron status, and the stage of the estrous cycle on core temperature ($P < 0.05$). Thus, the ability to thermoregulate is affected by the state of the estrous cycle more so in the ID than in the CN. Ovariectomy, therefore, tends to impair thermogenic performance to a greater extent in the ID animals than in CN, and ID animals at metestrus tended to maintain core temperature better than other ID groups.

The stepwise multiple regression analysis revealed that hematocrit and IBAT deiodinase activity were the best predictors of core temperature at 5 hours ($P < 0.0001$ and $P < 0.002$, respectively). Plasma thyroxine was also a significant predictor ($P < 0.05$) of final core temperature. The regression r^2 with these variables included in the model was 0.497. Due to differences in body weight between ID and CN animals, we have plotted change in core temperature (ie, $Temp_{5h} - Temp_{0h}$) versus body weight (*Figure 4*). These data demonstrate the lack of a specific effect of body weight (within ID and CN groups) on ability to thermoregulate.

Plasma T_4 concentrations at the end of cold exposure were unaffected by either iron status or the estrous cycle, while plasma T_3 concentrations and the $T_3 : T_4$ ratio were significantly lower in ID animals than CN (*Table 4*).

Liver thyroxine monodeiodinase activity was sig-

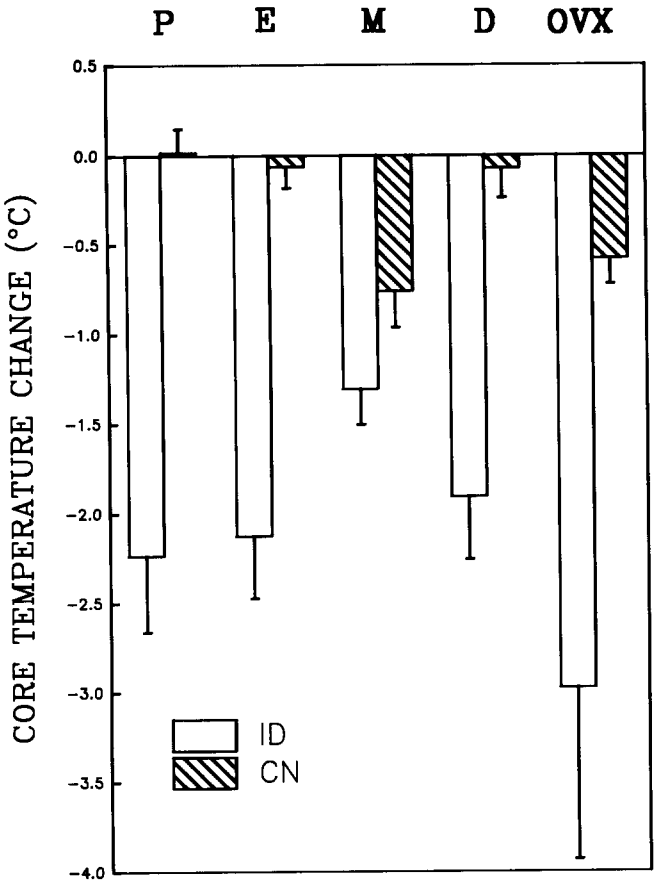


Figure 3 Core temperature change during acute cold exposure in iron-deficient (ID) and control (CN) rats during each stage of the estrous cycle. Data presented are mean ± standard error of the mean. The interaction of iron status and stage of cycle was significant ($P \leq 0.05$). P, proestrus; E, estrus; M, metestrus; D, diestrus; OVX, ovariectomized; TEMP, temperature.

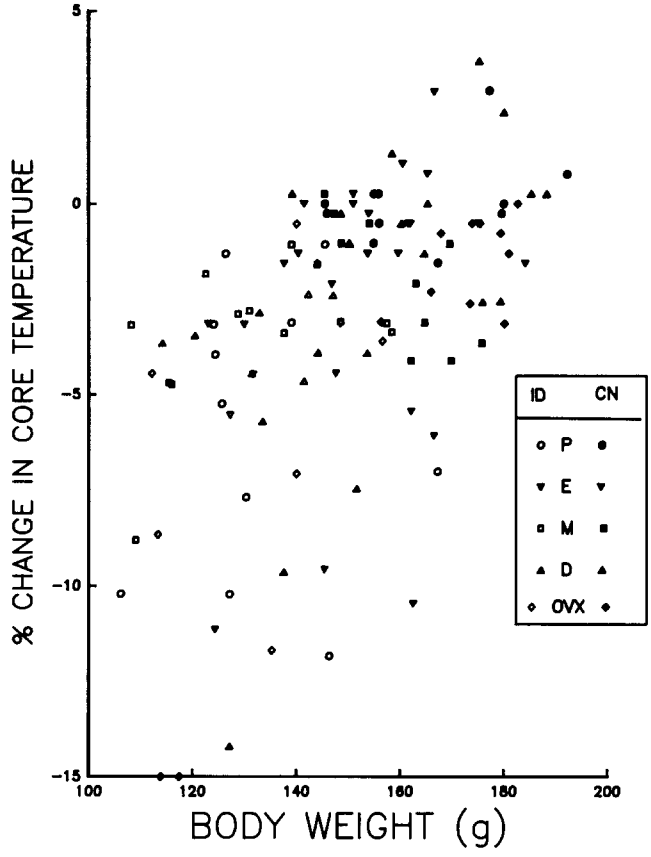


Figure 4 Relationship between body weight in grams and change in core temperature after a 6-hr cold stress in iron-deficient (ID) and control (CN) rats. P, proestrus; E, estrus; M, metestrus; D, diestrus; OVX, ovariectomized; TEMP, temperature.

nificantly lower in ID animals than in CN (*Figure 5*), but was unaffected by the estrous cycle. Brown adipose tissue deiodinase activity was unaffected by iron status or the estrous cycle (*Figure 5*).

Discussion

The present study was designed to assess the effects of acute cold stress on thyroid hormone metabolism in iron-deficient female rats. It demonstrates that iron-deficient female rats cannot maintain core temperature when cold stressed, and there is an interactive effect of the reproductive cycle or core temperature during acute cold exposure. Specifically, ID-P, ID-E, and ID-OVX animals have the largest decline in core temperature throughout a 6-hour cold stress (*Figure 3*). Studies of the thermogenic effects of the ovarian steroids have found estradiol to be a stimulating factor,[8,9] and plasma concentrations of estradiol are lowest during metestrus.[35] Thus, it is noteworthy that the CN-M animals tend to have a larger decline in core temperature during this phase of the estrous cycle, while ID animals actively maintain core temperature best during metestrus. These data suggest that iron-deficient animals do not respond to thermogenic effects of the ovarian steroids as do controls. This confirms results from ovariectomized iron-deficient rats at room temperature treated with estradiol and/or progesterone.*

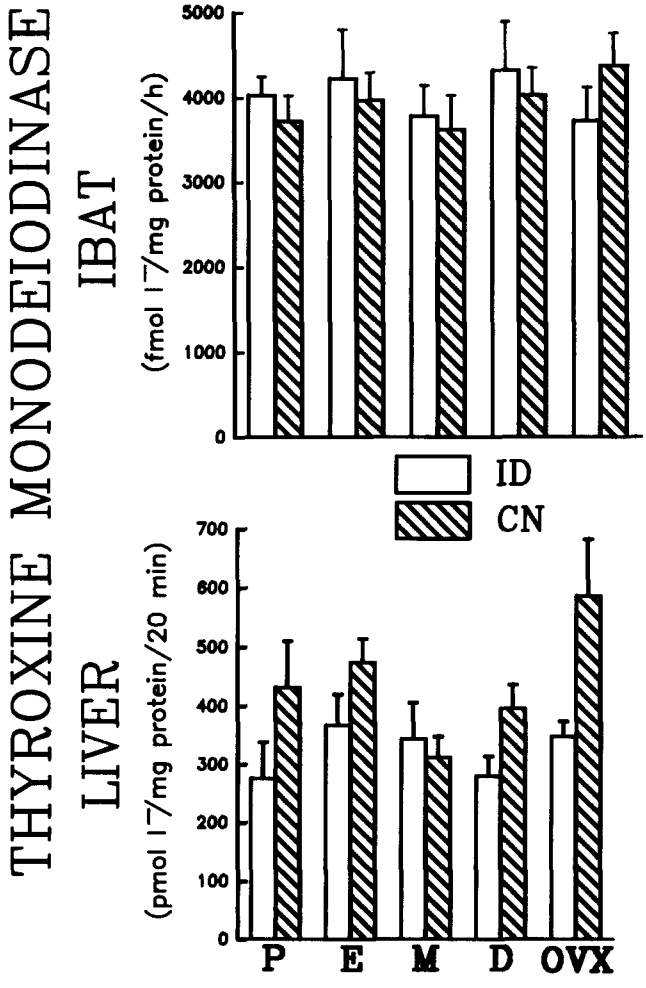
While the ID female rats had significantly lower core temperatures following acute cold stress, only one animal (an ID-OVX) had a core temperature below 30° C (final temp = 26.4° C). This is in contrast to data from ID male rats in which many animals (~50%) had to be removed from the cold because of rectal temperatures falling below 30° C.[22,23] While the obvious explanation for this phenomenon could be sex-related differences in body fat content and distribution, this does not appear to be the case. If these sex-related compositional differences are the primary cause of the thermoregulatory defect, one would expect a difference in response to cold between male and female CN rats. Also, studies comparing body composition of iron-sufficient and iron-deficient male rats have failed to demonstrate an effect of iron deficiency on percent body fat.[36] Some questions, however, still remain concerning iron-deficient female rats. Are

*Smith, S.M., Deaver, D.R., and Beard, J.L. Unpublished observations.

Table 4 Plasma thyroid hormones in ID and CN animals after a 6-hour cold stress

	(N)	Plasma thyroxine (nmol/L)	Plasma triiodothyronine (nmol/L)	Plasma T_3:T_4 ratio
Iron-deficient				
P	(11)	25.0 ± 5.8	1.27 ± 0.21	0.077 ± 0.023
E	(12)	26.5 ± 4.7	0.84 ± 0.10	0.038 ± 0.006
M	(13)	24.8 ± 5.0	1.00 ± 0.14	0.063 ± 0.017
D	(14)	22.2 ± 3.0	0.99 ± 0.12	0.057 ± 0.011
OVX	(8)	16.7 ± 1.0	0.75 ± 0.15	0.047 ± 0.010
Overall	(58)	23.4 ± 2.0	0.98 ± 0.07	0.057 ± 0.007
Control				
P	(11)	23.2 ± 3.7	1.68 ± 0.17	0.095 ± 0.019
E	(14)	31.0 ± 9.9	1.80 ± 0.08	0.097 ± 0.014
M	(10)	25.6 ± 3.4	1.67 ± 0.14	0.072 ± 0.009
D	(15)	27.7 ± 6.3	1.43 ± 0.09	0.074 ± 0.009
OVX	(10)	30.1 ± 3.7	1.45 ± 0.12	0.055 ± 0.009
Overall	(60)	27.7 ± 2.9	1.60 ± 0.05	0.080 ± 0.006
Analysis of variance table				
Iron status		NS	0.001	0.01
Estrous cycle		NS	NS	NS
Iron × cycle		NS	NS	NS

ID, iron-deficient; CN, control; P, proestrus; E, estrus; M, metestrus; D, diestrus; OVX, ovariectomized.
All data are presented as mean ± SEM.



body fat content and adipose tissue distribution altered in iron deficiency in females and, if so, does this cause the impaired ability to thermoregulate in these animals? There also does not seem to be a relationship between body size and change in core temperature (*Figure 4*) within ID or CN groups. This further supports a metabolic, as opposed to a body size/composition, cause of thermogenic impairment in iron deficiency.

Cold stress has removed a previously observed effect of the estrous cycle on the plasma thyroid hormones.* This suggests that although the reproductive cycle does affect thyroid hormone metabolism, environmental stressors can override this ovarian cycle influence. The lower concentration of plasma T_3 in the iron-deficient animals, despite unaltered thyroxine concentrations, reflects an impaired conversion of T_4 to T_3. The interactive response of cold exposure and iron status on core temperature is interesting in light of the lack of an effect of the estrous cycle on thyroid hormone variables. It is clear that the ovarian cycle affects thermoregulatory performance in the ID animals (as exemplified by the response of the ID-OVX group); however, this is not reflected by changes in

*Smith, S.M., Deaver, D.R., and Beard, J.L. Thyroxine 5'-deiodinase and metabolic rate in iron deficiency: effect of the estrous cycle. *FASEB J.* 4:A1159 (abstract #5736), 1990.

Figure 5 Liver and IBAT thyroxine monodeiodinase in iron-deficient (ID) and control (CN) rats during each stage of the estrous cycle and in ovariectomized (OVX) ID and CN rats. P, proestrus; E, estrus; M, metestrus; D, diestrus.

plasma thyroid hormones or tissue deiodinase activity. Whether this reflects an insensitivity of the measures of thyroid status or an effect of the ovarian cycle independent of these indices of thyroid metabolism remains to be determined.

It is interesting that in the present study the iron-deficient animals have lower plasma T_3 concentrations when cold exposed than do controls, while also having lower hepatic deiodinase activity, as would be expected. This was not the case, however, in animals raised at 24° C, where the relationship of deiodinase activity and plasma thyroid hormones was different than expected; ie, increases in deiodinase activity were not related to increases in plasma T_3.* We hypothesize that there is an increased sensitivity of the thyroid hormone system, as measured in vitro, when externally stressed (ie, cold exposure). Thus, the lack of a relationship noted in rats raised at 24° C may reflect an in vitro insensitivity of the deiodinase and/or plasma thyroid measurements. Studies of plasma and tissue thyroid hormone kinetics are currently underway to investigate this further.

In conclusion, we have shown that iron deficiency and the estrous cycle interact to affect thermoregulatory capacity in female rats. This apparently is the result of a defect in the ability of the thyroid hormone system to react to cold exposure in ID animals; however, the precise role of the ovarian cycle and ovarian steroids on these thyroid indices requires further examination.

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